New York State Department of Health - Wadsworth Center
Laboratory of Environmental Biology
NYS ELAP Laboratory ID 10765
Division of Environmental Health Sciences
Albany, New York

NYS DOH LEB-608

Identification of *Escherichia coli* in Medical Marijuana Products
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1.0. Scope and Application

1.1. This method NYS DOH LEB-608, Identification of *Escherichia coli* in Medical Marijuana Products, describes methods for detecting and identifying *Escherichia coli* (ELAP ID 9988) in samples of medical marijuana products as required in Title 10 (Health), Subpart 55-2.15 and Chapter XIII, Section 1004.14 of the official Compilation of Codes, Rules, and Regulations, of the State of New York.

1.2. This protocol describes methods for detecting and identifying *Escherichia coli* in medical marijuana samples. It is used as a follow-up to NYS DOH LEB-604 section 9.2, or section 9.7 (after verification of growth), and applies to sample enrichments showing growth in Trypticase Soy Broth at 30-35°C.

1.3. Protocols for the identification of these organisms in samples of medical marijuana products can be found in the NYS DOH LEB-600 series. See Medical Marijuana Microbial Testing Plan flowcharts.

2.0. Summary of the Method

2.1. Medical marijuana samples showing growth in Trypticase Soy Broth at 30-35°C are transferred to MacConkey Broth and incubated at 42-44°C for 24-48 hours. Samples that show growth in MacConkey Broth are subcultured onto MacConkey Agar and incubated at 30-35°C for 18-72 hours. Bacterial colonies are transferred to Trypticase Soy Agar plates and identified using API® 20E identification strips. Samples producing bacterial colonies on MacConkey Agar that are identified as *E. coli* are reported as positive.

3.0. Definitions

3.1. TSB stands for Trypticase Soy Broth
3.2. TSA stands for Trypticase Soy Agar
3.3. MCB stands for MacConkey Broth
3.4. MCA stands for MacConkey Agar

4.0. Health and Safety Warnings

4.1. Microbiological analyses involve the culturing of potentially pathogenic organisms.

4.1.1. All microbiologically contaminated materials, including media, shall be autoclaved after use.

4.1.2. Laboratory equipment and benches shall be disinfected using either Envirocide®, 10% bleach, or 70% ethanol before and after use.

4.1.3. Mouth pipetting is prohibited.

4.1.4. All accidents, particularly those which may result in infection, shall be reported according to laboratory specific procedures.

4.1.5. The analyst must be familiar with any possible hazards from the reagents and standards used for sample preparation and analysis.

4.1.6. Laboratory safety procedures shall be followed at all times. Regulations required by federal, state and local government agencies shall be implemented and followed.
4.1.8. The analyst must be familiar with any possible hazards from the reagents and standards used for sample preparation and analysis.

4.1.9. Always follow guidelines listed in safety data sheets (SDS) for proper storage, handling, and disposal of samples, solvents, reagents, and standards. SDSs are located within the laboratory. These guidelines must be made available to all personnel involved in microbiological analyses.

4.1.10. Appropriate lab coat, safety glasses and gloves must be worn when performing standard or sample preparations, working on instrumentation, disposing of waste, and cleaning laboratory equipment.

5.0. Shipping Conditions, Receiving, Preservation and Storage

5.1. Sample Shipping Conditions

5.1.1. The medical marijuana products from the Registered Organizations (ROs) are shipped as per manufacturer’s specifications and must adhere to all regulatory requirements.

5.2. Sample Receipt

5.2.1. Medical marijuana products from the RO are received, verified and documented ensuring that method, regulatory and Accreditation Body requirements are met.

5.3. Method Holding Times

5.3.1. This procedure is initiated upon completion of the Microbial Presence/Absence Test for Medical Marijuana Samples method (see NYS DOH LEB-604, section 9.2).

5.4. Preservation

5.4.1. Presence-Absence test aliquots that are presumptive positive for aerobic bacteria are stored refrigerated until it has been determined that they are not needed for additional microbiological evaluation.

5.5. Storage

5.5.1. If storage is required prior to analysis, isolates or archived plates are stored refrigerated until it has been determined that they are not needed for additional microbiological evaluation.

6.0. Interferences

6.1. Some components of medical marijuana products, e.g., ethanol, may inhibit the growth of microorganisms.

7.0. Apparatus and Materials

7.1. Equipment

7.1.1. Incubator, set at 30.0-35.0°C
7.1.2. Water bath, set at 42.0-44.0°C
7.1.3. Automatic pipettors and sterile aerosol-resistant micropipette tips
7.1.4. Sharpie or equivalent
7.1.5. Disposable sterile inoculating loops, 10µL
7.1.6. Disposable sterile inoculum spreader, or equivalent
7.1.7. Biosafety cabinet with HEPA filter

7.2. Reagents and Chemicals
7.2.1. MCB, 100mL bottles. Ensure that the formulation is in agreement with that specified by USP.
7.2.2. MCA, 15 x 100mm plates. Ensure that the formulation is in agreement with that specified by USP.
7.2.3. TSA, 15 x 100mm plates. Ensure that the formulation is in agreement with that specified by USP.
7.2.4. Disinfectants such as Envirocide® (Fisher Scientific cat. no. 19898220), 10% Bleach and/or 70% ethanol.

7.3. Forms
7.3.1. *E. coli* Identification Result Sheet (e.g., LEB-RS-608A).
7.3.2. API® 20E Identification Results Sheet
7.3.3. Walk-In Temperature Record
7.3.4. Cold Room Temperature Record
7.3.5. Refrigerator Temperature Record

8.0. Quality Control/Assurance
8.1. Method Detection Limits
8.1.1. Method Detection Limits are product-specific and are determined in accordance with relevant standards, regulations and Accreditation Body requirements.

8.2. Calibration and Standardization
8.2.1. Incubator temperatures shall be observed and recorded twice daily, separated by at least 4 hours.
    8.2.1.1. Temperature of the 30.0-35.0°C walk-in is recorded. If the incubator temperature does not stay within 30.0-35.0°C, laboratory-specific corrective actions are followed
    8.2.1.2. Analytical results may be invalidated if the incubator temperature exceeds 35.0°C, at the discretion of the laboratory.
8.2.2. Temperatures of the cold room and refrigerator are observed and recorded twice daily separated by at least 4 hours on either the Cold Room or Refrigerator Temperature Record.
    8.2.2.1. If the cold room or refrigerator does not stay within 1.0-8.0°C, laboratory-specific corrective actions are followed.
    8.2.2.2. The optimum temperature range for a cold room or refrigerator is 1.0-4.0°C
    8.2.2.3. If the cold room or refrigerator was in a defrost cycle at the time that the temperature was recorded, and the temperature does not reach 8.0°C, re-testing of media is not required.
    8.2.2.4. Media may be re-tested for quality, depending on the number of degrees and the amount of time that the cold room or refrigerator
temperature was out of compliance, at the discretion of the laboratory.

8.2.3. Water bath temperatures shall be observed and recorded twice daily, separated by at least 4 hours.

8.2.3.1. Temperature of the 42.0-44.0°C water bath is recorded.

8.2.3.1.1. If the water bath temperature does not stay within 42.0-44.0°C, laboratory-specific corrective actions are followed. Analytical results may be invalidated if the incubator temperature exceeds 44.0°C, at the discretion of the laboratory.

8.2.4. Max/min temperatures are recorded when twice-daily temperature measurements are not possible, such as on holidays and weekends.

8.2.5. Thermometers must be verified as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.2.6. The volumetric accuracy of automatic pipetters and serological pipettes is determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.2.7. The intensity and efficacy of the UV light in the biosafety cabinet is determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.2.8. Biosafety cabinets are certified annually.

8.3. Quality Control

8.3.1. Comparative recovery and sterility between lots of MCB, MCA, and TSA will be determined prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.3.2. Agar plates can be used for up to 2 weeks after the preparation date if stored refrigerated in plastic bags and in the dark.

8.3.2.1. Agar plates can be used after 2 weeks storage if ongoing QC demonstrates no loss in selectivity or growth promotion.

8.3.2.2. Analyses must be completed prior to the expiration date of the media and analyses must not be initiated on the day media expires.

8.3.3. Liquid media shall be stored in tightly-capped bottles in the dark at 4°C for up to 3 months from the date of preparation.

8.3.3.1. Liquid media can be used after 3 months storage if ongoing QC demonstrates no loss in selectivity or growth promotion.

8.3.3.2. Analyses must be completed prior to the expiration date of the media and analyses must not be initiated on the day media expires.

8.3.4. Sterility of disposable inoculation loops and spreaders are determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.
8.3.5. The use test for reagent water is performed annually, when cartridges are changed, or repairs are made to the deionized water systems.

8.4. Corrective/Preventive Actions
8.4.1. The laboratory will initiate non-conformances and/or corrective/preventive actions in accordance with laboratory-specific procedures and as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

9.0. Procedure
9.1. General
9.1.1. Aseptic technique is used for all procedures.
9.1.1.1. Aseptic technique can be found in a general microbiology textbook or on-line.
9.1.2. All work surfaces are disinfected prior to subculturing and colony identification.
9.1.3. Enrichment, subculturing, and colony identification are performed in a different location than sample preparation and initial sample analyses to prevent cross-contamination of incoming products.
9.1.4. All organisms isolated from turbid EEBM enriched sample must be identified regardless of if they are a regulated organism.

9.2. Enrichment in MacConkey Broth (MCB)
9.2.1. For each turbid TSB sample enrichment produced according to NYS DOH LEB-604 section 9.2., remove two 100mL aliquots of MCB from the cold room and warm to room temperature.
9.2.2. Inoculate 1mL from the turbid TSB sample enrichment into a 100mL MCB aliquot.
9.2.3. Inoculate 1mL from the corresponding turbid TSB matrix spike into a 100mL MCB aliquot.
9.2.3.1. If there isn’t a corresponding matrix spike, inoculate the MCB with the an isolated colony of *E. coli* ATCC 8739 from the monthly transfer plates as a positive control.
9.2.4. Shake to mix and incubate at 42.0-44.0°C for 24-48 hours.
9.2.5. After incubation, record the results of MCB sample enrichments either as “Y” for growth-positive (turbid) or “N” for negative (no turbidity) in the “MCB Sample Result” section on the *E. coli* Identification Results Sheet (e.g., LEB-RS-608A).
9.2.5.1. Record any color change of the medium.
9.2.5.1.1. Growth of *E. coli* in MCB is typically paralleled by a change in medium color from red/purple to yellow.
9.2.5.2. If the sample result is positive (turbid), proceed to 9.3.
9.2.5.3. If the sample result is negative (no turbidity), the sample is negative for the presence of *E. coli*.
9.2.5.4. If the turbidity in the TSB was confirmed as growth in LEB-604 section 9.7, but there was no growth in the MCB, proceed to 9.5 to identify any organisms isolated on nonselective agar plates from NYS DOH LEB-604 section 9.7.

9.2.6. After incubation, record the results of either the matrix spike or positive control MCB either as “Y” for growth-positive (turbid) or “N” for negative (no turbidity) in the “MCB M.S./P.C, Result” section on the *E. coli* Identification Results Sheet (e.g., LEB-RS-608A).

9.2.6.1. Record any color change of the medium.

9.2.6.2. If the sample result is negative in MCB, it is not necessary to continue with subculturing either the matrix spike or positive control.

9.2.6.3. If the sample and matrix spike enrichment results are negative in MCB, turbidity in the initial P/A TSB may have been caused by matrix characteristics. The test results are valid if the positive and negative controls for aerobic plate counts and mold plate counts meet QC criteria (see NYS DOH LEB-605).

9.2.6.4. If the matrix spike enrichment result is negative in MCB, and the positive and negative controls for aerobic plate counts and mold plate counts do not meet QC criteria (see NYS DOH LEB-605), results are considered invalid and the analyses must be repeated.

9.2.6.4.1. Additional testing using USP methods designed to decrease inhibitory effects of product that could result in growth in matrix spike samples may be undertaken using archived samples, at the discretion of the laboratory.

9.2.6.5. If a positive control is analyzed instead of a matrix spike, and the results are negative, the results are considered invalid and analyses must be repeated.

9.3. Subculture

9.3.1. For each turbid MCB sample enrichment produced according to section 9.2., remove three MCA plates from the cold room and warm to room temperature while drying in the biological safety cabinet.

9.3.2. Use an inoculating loop to streak the samples with turbid MCB onto two MCA plates for colony isolation.

9.3.3. Use a separate inoculating loop to streak the matrix spike or positive control MCB onto one MCA plate for colony isolation.

9.3.4. Once the MCA plates have dried, invert and incubate at 30.0-35.0°C for 18-72 hours.

9.3.4.1. Do not stack the plates more than four high.

9.3.5. After incubation, record the results for the sample MCA plates as “Y” for growth-positive (bacterial colonies are present) or “N” for negative...
(bacterial colonies are absent) in the “MCA Sample Result” section of the 
*E. coli* Identification Results Sheet (e.g., LEB-RS-608A).

**9.3.5.1.** If the sample is growth-positive on MCA, proceed to 9.4.

**9.3.5.2.** If there is no growth on the MCA plates, the sample is negative for *E. coli*.

**9.3.5.3.** If the turbidity in the TSB was confirmed as growth in NYS DOH LEB-604 section 9.7, but there was no growth on the MCA plates, proceed to 9.5 to identify any organisms isolated on nonselective agar plates from NYS DOH LEB-604 section 9.7.

**9.3.6.** After incubation, record the results for either the matrix spike or positive control MCA plate as “Y” for growth-positive (bacterial colonies are present) or “N” for negative (bacterial colonies are absent) in the “MCA M.S./P.C. Result” section of the *E. coli* Identification Results Sheet (e.g., LEB-RS-608A).

**9.3.6.1.** If the sample result is negative on MCA, it is not necessary to identify colonies on either the matrix spike or positive control plate that shows colonies having morphology typical of *E. coli* ATCC 8739.

**9.3.6.1.1.** Typical *E. coli* ATCC 8739 colonies are red/pink or purple and may be surrounded by a zone of bile precipitation (halo), depending on the medium manufacturer.

**9.3.6.2.** If the matrix spike result is negative on MCA, the test results are valid if the positive and negative controls for aerobic plate counts and mold plate counts meet QC criteria (see NYS DOH LEB-605).

**9.3.6.3.** If the matrix spike result is negative on MCA, and the positive and negative controls for aerobic plate counts and mold plate counts do not meet QC criteria (see NYS DOH LEB-605), results are considered invalid and the analyses must be repeated.

**9.3.6.3.1.** Additional testing using USP methods designed to decrease inhibitory effects of product that could result in growth in matrix spike samples may be undertaken using archived samples, at the discretion of the laboratory.

**9.3.6.4.** If a positive control is analyzed instead of a matrix spike, and the results are negative, the results are considered invalid and analyses must be repeated.
9.4.  Colony Identification

9.4.1. Select one or more well-isolated colonies having distinct morphologies from growth-positive sample and matrix spike/positive control MCA plates and record the sample sources and colony morphologies on the E. coli Identification Results Sheet (e.g., LEB-RS-608A).

9.4.1.1. If growth is confluent on the MCA plates, re-streak for isolation of individual colonies and proceed with 9.4.1.

9.4.2. For each colony being identified, remove one TSA plate from the cold room and warm to room temperature while drying.

9.4.3. Streak well-isolated colonies from the growth-positive sample MCA plates onto TSA plates for colony isolation.

9.4.3.1. Bacterial colonies can be selected from either of the two MCA sample plates prepared in section 9.5.

9.4.4. Select a well-isolated colony showing characteristics of E. coli ATCC 8739 from either the matrix spike or positive control MCA plate and streak onto TSA plates that have been warmed to room temperature and dried in a biological safety cabinet.

9.4.4.1. Record the sample source and colony morphology on the E. coli Identification Results Sheet (e.g., LEB-RS-608A).

9.4.4.2. If growth is confluent, re-streak for isolation of individual colonies and proceed with 9.4.4.

9.4.5. Once the TSA plates have dried, invert and incubate at 30.0-35.0°C for 18-24 hours.

9.4.5.1. Do not stack more than four high.

9.4.6. After incubation, use the growth on the TSA plates to perform a Gram stain following the instructions in NYS DOH LEB-613.

9.4.7. Record the results of the Gram stain on the E. coli Identification Results Sheet (e.g., LEB-RS-608A).

9.4.8. If the organisms are not gram-negative rods proceed to 9.5.

9.4.9. If the organisms are gram negative rods, use the growth on the TSA plates to proceed with the API® Identification Test Strip method to identify the organisms using the API® 20E Test Strips.

9.4.9.1. Attach all API® 20E Identification Results sheets to the E. coli Identification Results Sheet (e.g., LEB-RS-608A).

9.4.10. If the API® 20E Identification Test kit fails to identify the isolate proceed to 9.5.

9.5.  Identification of Non-Target Organisms

9.5.1. The identification of non-regulated bacterial contaminants is required.

9.5.2. In cases where there is growth of a non-regulated analyte(s), consultation with the NYS Medical Marijuana Program is required.
10.0. Data Acquisition, Reduction, Analysis, Calculations, Acceptance Criteria and Documentation

10.1. Record the accession number, analyst initials, MCB lot date, incubation start and end dates and times, MCA lot date, incubation start and end dates and times, TSA lot date, incubation start and end dates and times, colony morphology, source of colony (matrix spike, positive control, or sample), Gram stain results, colony identification and results of testing on the \textit{E. coli} Identification Results Sheet (e.g., LEB-RS-608A).

10.2. If the identified organism(s) is \textit{E. coli}, circle “Positive for \textit{E. coli}”, otherwise circle “Negative for \textit{E. coli}” on the \textit{E. coli} Identification Results Sheet (e.g., LEB-RS-608A).

10.3. If an identified organism(s) isn’t a regulated analyte, check the comment at the bottom of the form, and record the identified organism on the \textit{E. coli} Results Sheet (e.g., LEB-RS-606B).

10.3.1. The comment at the bottom of the results sheet is added as a note in the final report.

10.4. If an identified organism(s) is a regulated analyte that is not \textit{E. coli}, the sample is reported as positive for the regulated analyte, but a note is entered onto the report explaining that the organism was isolated from the aerobic bacteria presence/absence analyses.

10.5. If at any point in the analyses the matrix spike fails to grow in any medium, the test results are valid as long as the positive and negative controls for the aerobic plate counts and mold plate counts meet QC criteria (see NYS DOH LEB-605).

11.0. Method Performance

11.1. Demonstration of Capability

11.1.1. Prior to acceptance and use of this method for data reporting, a satisfactory initial demonstration of capability (DOC) is required. Thereafter, an ongoing DOC is to be performed annually.

11.1.2. An initial DOC shall be made prior to using any method, and at any time there is a change in instrument type, personnel or method or any time that a method has not been performed by the laboratory or analyst in a twelve (12) month period.

11.1.3. All DOCs shall be documented, and all data applicable to the demonstration shall be retained and readily available at the laboratory. Consult state regulations and standards for additional information on performing a DOC for microbiological contaminants.

11.1.4. Consult state regulations and standards for additional information on performing a DOC for microbiological contaminants.

11.2. Laboratory Detection Limits

11.2.1. Detection limits are determined in accordance with relevant standards, regulations and accreditation body requirements.
12.0. Waste Management/Pollution Prevention

12.1. It is the responsibility of the laboratory to comply with all federal, state and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions.

12.2. Bacterial/fungal cultures and contaminated or potentially contaminated disposable materials are disposed of in biohazardous waste cans and autoclaved prior to discarding.

12.3. Dispose of non-hazardous aqueous waste in the laboratory sink followed by flushing with tap water.

12.4. Dispose of glassware in appropriately labeled boxes. Be sure that, whenever possible, the glass has been thoroughly rinsed and is contaminant-free before disposal.

12.5. Consult federal, state and local regulations for additional information or for information on the disposal of products not described in this method.

13.0. References


13.4. TNI 2016 Standards – EL-V1M5-2016-Rev2.0: Microbiological Testing

13.5. API® 20E Test Strips Instructions for Use, bioMérieux

13.6. Title 10 (Health), Subpart 55-2.15 and Chapter XIII, Section 1004.14 of the official Compilation of Codes, Rules, and Regulations, of the State of New York.

13.7. NYS DOH LEB-604, Microbial Presence/Absence Test for Medical Marijuana Samples

13.8. NYS DOH LEB-605, Aerobic Bacteria and Mold Plate Counts for Medical Marijuana Testing

13.9. NYS DOH LEB-613, Identification of Thermophilic Actinomycetes in Medical Marijuana Products
14.0. Appendices – Forms

**E. coli Identification Results Sheet (LEB-RS-608A)**

Incubate MCB for 24-48 hours, MCA for 18-72 hours, and TSA 18-24 hours
(30-35°C Incubator and 42-44°C Water Bath)

<table>
<thead>
<tr>
<th>Accession Number:</th>
<th>Analyst Initials:</th>
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<tbody>
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Final Results (circle one):

- Negative for *E. coli*
- Positive for *E. coli*

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<thead>
<tr>
<th>MCB Start Date/Time:</th>
<th>Sample Growth/Color:</th>
<th>Y</th>
<th>N</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>M.S./P.C. (circle one) Growth/Color:</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>MCB Lot Date:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCA Start Date/Time:</td>
<td>Sample Growth:</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>MCA Lot Date:</td>
<td>M.S./P.C. (circle one) Growth:</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>TSA Start Date/Time:</td>
<td>Gram Stain Results:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSA End Date/Time:</td>
<td>API® 20E Start Date/Time:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSA Lot Date:</td>
<td>API® 20E End Date/Time:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram Stain Date/Time:</td>
<td>Stain Positive Control Result:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram Stain Lot/Exp Date:</td>
<td>Stain Negative Control Result:</td>
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Source (Sample, M.S., or P.C.), Colony Morphology and API® 20E Colony Identification

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
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</table>

All API® 20E Result Sheets (LEB-RS-614A) are attached. MCA=MacConkey Agar, MCB=MacConkey Broth, TSA = Trypticase Soy Agar, M.S. = matrix spike, P.C. = positive control plate used in lieu of a matrix spike. *E. coli* ATCC 9739, used as a matrix spike, is typically red/violet-purple with a purple halo on MCA.

If the contaminant is a non-regulated analyte(s), check below and fill in the organism(s) identified so the text will be entered as a note on the report: ___ (“+” for _____________________________________________________________________ (not ELAP-regulated analyte)"

Reviewed by:     Date:

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Empire State Plaza, Coming Tower, Albany, NY 12237 | health.ny.gov
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